The group B streptococcal alpha C protein binds $\alpha_1\beta_1$-integrin through a novel KTD motif that promotes internalization of GBS within human epithelial cells

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Group B Streptococcus (GBS) is the leading cause of bacterial pneumonia, sepsis and meningitis among neonates and a cause of morbidity among pregnant women and immunocompromised adults. GBS epithelial cell invasion is associated with expression of alpha C protein (ACP). Loss of ACP expression results in a decrease in GBS internalization and translocation across human cervical epithelial cells (ME180). Soluble ACP and its 170 amino acid N-terminal region (NtACP), but not the repeat protein RR$_9$, bind to ME180 cells and reduce internalization of wild-type GBS to levels obtained with an ACP-deficient isogenic mutant. In the current study, ACP colocalized with $\alpha_1\beta_1$-integrin, resulting in integrin clustering as determined by laser scanning confocal microscopy. NtACP contains two structural domains, D1 and D2. D1 is structurally similar to fibronectin’s integrin-binding region (FnIII10). D1’s (KT)D146 motif is structurally similar to the FnIII10 (RG)D1495 integrin-binding motif, suggesting that ACP binds $\alpha_1\beta_1$-integrin via the D1 domain. The (KT)D146A mutation within soluble NtACP reduced its ability to bind $\alpha_1\beta_1$-integrin and inhibit GBS internalization within ME180 cells. Thus ACP binding to human epithelial cell integrins appears to contribute to GBS internalization within epithelial cells.

INTRODUCTION

Group B Streptococcus (GBS; Streptococcus agalactiae) is the leading cause of neonatal bacterial invasive diseases (Arisoy et al., 2003; Manning et al., 2004). The organism colonizes the vagina, rectum and urethra of 10–35 % of adults without causing disease. Neonates acquire GBS either in utero or during birth, aspirating GBS-containing fluid. GBS enters the lungs, binds to and invades the alveolar epithelial cells, and enters the blood, allowing the organisms to infect multiple organs. Invasive disease occurs within hours of birth, with 4–6 % mortality (Baker & Edwards, 1995; Schrag et al., 2000). In a survey in the USA, the incidence of early-onset disease (within the first week of life) declined by 65 % (from 1.7 per 1000 live births to 0.6 per 1000) from 1993 to 1998, due to intrapartum antimicrobial prophylaxis (Schrag et al., 2000) and stabilized at 0.35 per 1000 in 2004. The incidence of late-onset disease (>1 week to 3 months of age) remained stable, with an average of 0.35 per 1000 live births during the same time period (CDC, 1997, 2005).

GBS was more recently found to be pathogenic in the elderly, and in adults with underlying medical conditions, including diabetes (Dahl et al., 2003; Jackson et al., 1995; Tyrrell et al., 2000). Adult invasive GBS diseases result in bacteremia, meningitis, skin and soft-tissue infection, and bone and joint infections. The rate of invasive diseases is significantly higher in neonates than in adults. However, the case fatality rates are greater in adults (Schuchat, 1999), with the incidence of GBS disease increasing with advanced age (Farley et al., 1993). Despite the clinical importance of these diseases, little is known about the events that lead up to GBS invasion and its virulence factors. Several studies suggest that surface proteins play a major role in GBS binding to and invasion of human mucosal surfaces (Bulgakova et al., 1986; Tamura et al., 1994). We previously reported that the alpha C protein (ACP) is one such protein (Bolduc et al., 2002).

ACP is the prototype of a family of surface proteins known as alpha-like proteins (Alps) found on 90 % of GBS clinical
isolates, as well as other Gram-positive organisms (Lachenauer et al., 2000; Shankar et al., 1999; Stalhammar-Carlemalm et al., 1999; Turner et al., 2003). ACP consists of an N-terminal domain (NtACP; 170 amino acids) followed by a variable number of tandem repeats (82 amino acids each) and a C-terminal domain (45 amino acids) containing an LPXTG peptidoglycan-anchoring motif (Michel et al., 1992). Nearly all strains of serotype Ia, II and most of Ib express ACP on their surface (Madoff et al., 1991). In addition, some human serotype III, IV and NT strains and bovine V, VII and non-typable strains contain the ACP gene (bca) (Credi et al., 2004).

The biological function(s) of the Alp family of proteins remains unclear (Lindahl et al., 2005). Deleting bca attenuates the virulence of GBS in the neonatal mouse model by approximately sevenfold (Li et al., 1997). The lack of ACP expression in vitro reduces the ability of GBS to invade ME180 cells, a human cervical carcinoma epithelial cell line (Bolduc et al., 2002). ME180 cells resemble stratified eccoecutical squamous epithelial cells and are an established cellular model for studying group A and B streptococcal binding and invasion (Soriani et al., 2006; Stalhammar-Carlemalm et al., 1999; Sykes et al., 1970). The group A Streptococcus Alp R28 promotes binding to ME180 cells and is essentially identical to GBS Alp3 (Lachenauer et al., 2000). Similarly, adherence of Enterococcus faecalis, lacking expression of the Alp Esp, to urinary epithelium is reduced (Shankar et al., 2001). Together, these data provide strong evidence for the role of Alps in the adherence to and invasion of epithelial cells by Gram-positive micro-organisms.

We solved the tertiary structure of NtACP by X-ray crystallography (Auperin et al., 2005). NtACP can be further divided into two structurally distinct domains, D1 and D2. D1, the more distal (amino-terminal) portion, consists of a beta sandwich with strong structural homology to fibronectin’s integrin-binding domain (FnIII10). D2 consists of three antiparallel alpha helix coils containing a portion of the glycosaminoglycan (GAG)-binding domain. A partial putative GAG-binding site was mapped within domain 2 (D2), adjacent to the repeat region, consistent with our data showing that NtACP binds to heparin and GAGs only when it is covalently associated with the adjacent repeat region (Baron et al., 2004). However, exogenous soluble NtACP alone (without the repeat region) can still competitively bind to ME180 cells and reduce internalization and translocation of GBS (Bolduc et al., 2002), suggesting that NtACP binds to another non-GAG receptor. The structural data also revealed a potential integrin-binding site located on the most distal amino-terminal portion of NtACP (domain 1; D1), opposite the GAG-binding site on D2. We sought to determine if ACP binds to integrins present on the surface of ME180 cells and, if so, to identify the specific integrin heterodimeric molecules. Secondly, we tested the hypothesis that the binding of ACP’s D1 domain to integrin plays a role in GBS invasion.

METHODS

Bacterial strains and epithelial cell line. GBS type Ia/C (α, β+) strain A909 and the ACP-deficient mutant JL2053 have been described (Li et al., 1997). A909 was obtained from Dr Rebecca Lancefield (Lancefield et al., 1975). JL2053 was obtained from Dr Jing Li. ME180 cells (HTB33) (Sykes et al., 1970) were purchased from the American Type Culture Collection (Manassas, VA, USA).

Cloning and site-directed mutagenesis of ACP-specific proteins. The amino acid sequence of ACP is in the NCBI Protein Database under accession number AAA6848 (Auperin et al., 2005; Michel et al., 1992). The DNA sequence encoding the ACP (pCL1), NtACP (pDEK14), repeat region RR’ (pDEK14 RR’), and ACR has been cloned previously (Auperin et al., 2005; Bolduc et al., 2002; Gravekamp et al., 1996; Kling et al., 1997). The aspartic acid residue (Asp144) of the recombinant NtACP was substituted with an alanine residue (D146A) using the Quik-Change site-directed mutagenesis kit (Stratagene) according to the manufacturer’s instructions. Forward primer 5′-CCA CAT GTA AAG ACT GCT GGA CAA ATT GAT-3′ and reverse primer 5′-ATC AAT TTG TGC AGT CTT TAG ATG GAG-3′ and the pDEK14 template were used, resulting in the pGB3 construct. The underline nucleotides indicate the mutated codon.

The DNA sequence encoding the D2 region (Ser146-Leu225) was PCR amplified from pDEK14 using forward primer 5′-C GTG AGC ACA TTG AGG GAT AGG GAT ATT GAA-3′ and reverse primer 5′-ATC AAT TTG TGC AGT CTT TAG ATG GAG-3′. The 209 bp PCR product was cloned into pCR2.1-TOPO, transformed into Top10 cells (Invitrogen), excised with Nhel and EcoRI, and recloned into pTrcHisA. The resulting construct is pGB4. The DNA sequence encoding the Inv497 protein was PCR amplified from pRI203 (Hamburger et al., 1999) using forward primer 5′-C GTG AGC AGT GTC ACC GTG CAG CAA ATT GAT-3′ and reverse primer 5′-TTG GCC CAA TAC CAA TTT CTC CTA ATC-3′. The 1494 bp PCR product was cloned into pCR2.1-TOPO and transformed into Top10 cells. The plasmid was isolated and digested with Nhel, EcoRI and SphI. The inv497 Nhel/EcoRI fragment was gel purified and cloned into pTrcHisC. The resulting construct pGB5 was transformed into Escherichia coli strain BLR.

Protein expression and purification. Plasmids pET200/D-TOPO::D2-R, pDEK14 and pGB3 were transformed into E. coli BL21(DE3). Plasmids pGB4, pGB5 and pCL1 were transformed into E. coli BLR. Plasmid pET24RR was transformed into BL21(DE3). Expression and purification of each protein was performed as previously described (Bolduc et al., 2002). Briefly, protein expression was induced with 1 mM IPTG. The cells were harvested, lysed and the lysates loaded onto an 18 ml Fractogel (M) (Novagen) column charged with 50 mM NiSO4. Fractions containing recombinant protein were pooled and dialysed in 20 mM HEPES, pH 7.2.

ME180-binding assay. ME180 cells were grown to confluence in 96-well titre plates (Falcon Microtest 96 353072) in RPMI 1640 with 1-glutamine containing 10% Newborn Bovine Serum (26010-074; Invitrogen), 100 U penicillin ml−1 and 100 μg streptomycin ml−1 (15140-122; Invitrogen) at 37 °C with 5% CO2. Purified ACP, NtACP, NtACP D146A, D2-R and RR’ were biotin-labelled using the EZ-Link Sulfo-NHS-Biotinylation kit (Pierce). The moles of biotin per mol protein were determined by the automatic HABA calculator available on the Pierce website (http://www.piercenet.com). The proteins were incubated with the cells for 4 h at 37 °C with 5% CO2. Unbound proteins were removed by gently washing three times with Dulbecco’s phosphate-buffered saline (PBS), pH 7.1 (Gibco) containing 0.1 mM CaCl2 (PBS + CaCl2). The cells were fixed with 2% paraformaldehyde in PBS + CaCl2 and incubated in 0.5% BSA in PBS + CaCl2 for 18 h at 4 °C. Bound proteins were detected with
avadin–peroxidase conjugate (1:100 000; Sigma) in 0.5 % BSA in PBS + CaCl₂ and developed with o-phenylenediamine tablets (Sigma P-6662) dissolved in phosphate/citrate buffer. The absorbance was read at 490 nm and 650 nm and recorded as ΔΔ. The molar ratios of biotin to ACP, NtACP, NTACP D146A, D2-R, and RR’ were 2:1, 1:1, 1:1, and 1:1, respectively. Therefore, the values obtained for ACP were divided by 2 to compare with the values obtained for the other proteins.

Inhibition assays were performed similarly with the exception that 0–500 μM unlabelled NtACP, NTACP D146A and RR’ was added to the wells containing 20 μM biotinylated NtACP. The percentages of inhibition of bound biotinylated NTACP were calculated with the formula [(uninhibited ΔΔ – inhibited ΔΔ)/uninhibited ΔΔ]×100. Statistical analysis was performed using the paired t-test (http://www.graphpad.com/quickcalcstest1.cfm). A P value equal to or less than 0.050 is considered statistically significant.

**α,β1-integrin-binding assay.** Purified human αvβ1-integrin (5 μg ml⁻¹; Chemicon International) in 50 mM carbonate/bicarbonate buffer, pH 9.6, was allowed to bind to the wells of 96-well titre plates (NUNC flat-bottom MicroWell plate, MaxiSorp surface-treated) during an incubation of 2h at 37°C. The wells were blocked with 2 % BSA in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 overnight at 4°C. Antibodies to human αv or β1-integrin subunits were added to the wells along with 0–20 μM of NTACP, NTACP D146A mutant, RR’ or BSA, in 2 % BSA in 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 to allow binding to the immobilized αvβ1-integrin for 1h at room temperature. Antibodies used were: 1:400 dilution of rabbit anti-human αv-integrin antibodies (AB1934; Chemicon International), 1:400 dilution of rabbit anti-human β1-integrin antibodies (AB1952; Chemicon International) and 1:500 dilution of mouse anti-human αv-integrin 1-domain monoclonal antibody (MAB1973Z; Chemicon International). The wells were washed with 50 mM Tris/HCl pH 7.4, 150 mM NaCl, 2 mM MgCl₂, 0.1 mM CaCl₂, 0.1 % Triton X-100 to remove unbound ACP and antibodies. Binding of rabbit polyclonal antibodies was detected by adding a 1:500 dilution of anti-rabbit IgG-peroxidase conjugated antibody (A6154; Sigma) followed by OPD substrate (P6662; Sigma). Absorbance was recorded at 490 nm and 650 nm. The results are reported as ΔΔ.

**Confocal microscopy.** ME180 cells were grown in 0.5 ml RPMI medium 1640 with l-glutamine, 10 % fetal calf serum, 25 mM HEPES, 0.4 M CaCl₂ on a 12 mm diameter transwell-COL (3.0 μm pore size, Costar) membrane suspended in wells of a 12-well plate containing 1.5 ml of similar medium, as previously described (Bolduc et al., 2002). ACP, BSA and Inv497 (Hamburger et al., 1999) were labelled with AlexaFluor 568 using the AlexaFluor 568 Protein Labelling kit (Molecular Probes; A-10238). Aliquots of 0.25 ml of 1 μM labelled proteins were added to the apical side of the cells for 4h at 37°C with 5 % CO₂. Unbound protein was removed by gently washing the cells three times with PBS. The cells were fixed by washing the cells three times with PBS. The cells were permeated by incubating the membrane in 200 μl of 0.1 % Triton X-100 in PBS for 10 min at 22°C. The cells were washed once in PBS and incubated in 0.5 % BSA in PBS for 30 min at 22°C to block non-specific binding. Primary antibodies to α-integrins (Chemicon International; ECM445) and β-integrins (Chemicon International; ECM440) were diluted 1:500 in 0.5 % BSA in PBS. MAB1951Z (mouse anti-human integrin β1) was diluted in 0.5 % BSA in PBS containing 1 M CaCl₂, as its epitope binding is Ca²⁺-dependent; 200 μl of the antibodies was added to the cells for 1h at 22°C. The cells were washed three times with PBS containing 1 M CaCl₂.

Internalization assay. The assay was performed as previously described (Bolduc et al., 2002). Briefly, ME180 cells were grown to confluence in 24-well culture plates containing 1 ml RPMI medium (RPMI 1640 with l-glutamine, 10 % fetal calf serum, 25 mM HEPES). On the day of the assay, the medium was removed from the wells and 0.4 ml fresh RPMI medium was added to the cells. Soluble proteins (0.1 ml of NtACP, NTACP D146A, D2, D2-R, RR’ and BSA) were added to the cells 1h before adding 0.5 ml of 2×10⁸ c.f.u. ml⁻¹ of A909 and JL2503 (m.o.i.=5 bacteria cell⁻¹). The cells were incubated at 37°C with 5 % CO₂ for 2h. The monolayers were washed three times with PBS before adding 1 ml RPMI medium containing 100 μg gentamicin ml⁻¹ and 5 μg penicillin G ml⁻¹ per well for 2h at 37°C with 5 % CO₂. The cells were then washed with PBS, lysed in 0.4 ml of 0.025 % Triton X-100, and serial dilutions of the epithelial lysates plated on THA plates. The percentage of internalized bacteria was calculated as [(c.f.u. per well after antibiotic treatment)/c.f.u. originally added to the well]×100. Percentage inhibition was calculated as [(c.f.u. of internalized GBS)/(c.f.u. of original inoculum)]/[c.f.u. of internalized GBS un inhibited]/(c.f.u. of original inoculum of GBS uninhibited) × 100. Statistical analysis was performed using the unpaired t-test (http://www.graphpad.com/quickcalcstest1.cfm). A P value equal to or less than 0.050 is considered statistically significant.

Translocation inhibition assay. The assay was performed as previously described with the additional step of preincubating the cell membranes with soluble protein before adding GBS (Bolduc et al., 2002). Before GBS strain A909 or JL2053 was added to the cells, the medium was removed from the top chamber and replaced with 0.1 ml of 1, 5, 10, or 20 μM of repeat protein (RR’), NtACP, ACP, or BSA in RPMI medium 1640 with l-glutamine, 10 % fetal calf serum, 25 mM HEPES, 0.4 M CaCl₂. The cells were incubated at 37°C for 1h. Without removing the soluble protein, 0.4 ml of approximately 2×10⁷ c.f.u. ml⁻¹ of GBS strain A909 or JL2053 was added to the top chamber, and the cells were incubated for up to 4h. *E. coli* DH5α served as a non-invasive control.

**RESULTS**

The D1 KTD motif is involved in NtACP binding to ME180 cells

ACP, NtACP and D2-R (a construct containing D2 and the first adjacent repeat region) (Fig. 1a) were previously determined to bind qualitatively to ME180 by confocal microscopy and flow cytometry (Baron et al., 2004; Bolduc et al., 2002). In the current study, the proteins were biotin-labelled and quantitatively assayed for their ability to bind
ME180 cells in an ELISA-based binding assay. The labelled proteins were allowed to interact with the cells for 4 h. The cells were then washed with saline to remove unbound protein, and the remaining proteins were detected with avidin conjugated to horseradish peroxidase. ACP and NtACP bound similarly to ME180 cells, whereas NtACP D146A and D2-R demonstrated less binding. RR' did not bind (Fig. 1b), suggesting that the observed binding of ACP to ME180 cells is through the NtACP region.

NtACP consists of two structurally distinct domains, D1 and D2. We sought to locate NtACP’s binding site(s) within one or both of these domains (Fig. 1c). Unable to express the isolated D1 region in a sufficient quantity, we chose to mutate D1 in an NtACP background and compare its ability to bind ME180 cells with that of wild-type NtACP. The integrin-binding activity of type III fibronectin (FnIII) requires the presence of the Arg^{1493}-Gly^{1494}-Asp^{1495} (RGD) motif on FnIII module 10 (FnIII10) and Arg^{1379} and Asp^{1373} on FnIII module 9 (FnIII9) (Hamburger et al., 1999). A structurally similar triad of charged residues required for integrin binding is present in Yersinia pseudotuberculosis Inv497. Inv497 is a non-RGD protein that competes with FnIII by binding to \( \alpha_{5}\beta_1 \)-integrin (Isberg et al., 2000). NtACP’s D1 region contains a K^{144}-T^{145}-D^{146} (KTD) motif, located within a loop region that is structurally analogous to the loop containing the RGD integrin-binding motif in FnIII10. D1 also contains residues Arg^{110} and Asp^{118}, forming a triangular arrangement of charged residues similar to those found in fibronectin and Y. pseudotuberculosis Inv497. If NtACP binds integrin, we hypothesize that a (KT)D^{146}A substitution (NtACP D146A) may reduce its ability to bind ME180 cells.

Biotinylated NtACP along with either unlabelled NtACP or NtACP D146A was added to the cells for 4 h. We hypothesize that the labelling of NtACP with biotin may have increased the protein’s ability to bind ME180 cells, since it required 25-fold more unlabelled NtACP to inhibit binding of biotin-labelled NtACP by 60% (Fig. 2). Purified D2 was also tested for its ability to inhibit NtACP binding to ME180 cells; the inhibition was substantially less than that seen with NtACP (\( P = 0.0204 \) at 250 \( \mu M \), \( P = 0.0054 \) at 500 \( \mu M \)). The data suggest that NtACP binds to an ME180 surface receptor via D1. The loss of inhibition due to a single mutation in the hypothetical integrin-binding KTD motif suggests that the receptor is an integrin heterodimer.

ACP binds \( \alpha_{5}\beta_1 \)-integrin

In order to identify the integrin heterodimer(s) that may bind ACP, we next characterized the integrin profile of ME180 cells. Individual \( \alpha \)- and \( \beta \)-integrin subunits were probed with antibodies specific to the subunits, followed with AlexaFluor 488-labelled secondary antibodies, and examined by confocal microscopy. The cells stained with equally strong intensity for \( \beta_1 \) and \( \beta_2 \) subunits (Fig. 3a). Staining for \( \beta_3 \), \( \beta_4 \) and \( \beta_5 \) subunits was near background levels and not detectable. ME180 cells also stained with
strong positive intensity for $\alpha_1$, $\alpha_2$ and $\alpha_3$ subunits, but not for $\alpha_4$, $\alpha_5$ and $\alpha_6$ subunits (Fig. 3b).

We sought to determine whether ACP colocalizes with the integrin subunits. ACP, Inv497 and BSA (all labelled with AlexaFluor 568) were added to a polarized, semipermeable layer of ME180 cells for 4 h and examined by confocal microscopy. Inv497 (the extracellular C-terminal 497 residues of invasin from *Y. pseudotuberculosis*) binds to $\alpha_1\beta_1$-integrin and was included as a control (Van Nhieu & Isberg, 1991). Both ACP and Inv497 colocalized with $\beta_1$ but not with $\beta_2$ subunits (Fig. 4a, b). Interestingly, the cells with the most intense staining for ACP also demonstrated the highest fluorescent staining for the $\beta_1$ subunit, with little to no staining for the $\beta_2$ subunit. One of two possible explanations is that the interaction between ACP and ME180 cells results in a clustering of $\beta_1$ subunits. The other possibility is that, upon interaction with ACP, $\beta_1$ expression increases while $\beta_2$ expression decreases. AlexaFluor 568-labelled BSA served as a negative control (Fig. 4c). ACP also colocalized with the $\alpha_1$ subunit (Fig. 4d) and not with $\alpha_2$ or $\alpha_3$ subunits. The $\alpha_1$ and $\beta_1$ subunits colocalize with ACP on ME180 cells (Fig. 4e), identifying the $\alpha_1\beta_1$-integrin heterodimer known as the very late antigen 1 (VLA1; CD49a/CD29; laminin and collagen receptor) (Hemler *et al.*, 1986).

Direct binding of NtACP, NtACP D146A, RR', D2-R (at 10 $\mu$M) and BSA to $\alpha_1\beta_1$-integrin was measured by ELISA. Purified $\alpha_1\beta_1$-integrin was used to coat the wells of a 96-well microtitre plate. Mono- and polyclonal antibodies to the $\alpha_1$ subunit were added to the wells containing various concentrations of the proteins. The proteins’ abilities to inhibit binding of the antiserum were recorded as percentage inhibition. NtACP inhibited the $\alpha_1$ subunit polyclonal antibodies from binding, while NtACP D146A showed significantly less inhibition ($P=0.0140$ at 5 $\mu$M, $P=0.0042$ at 10 $\mu$M and $P=0.0243$ at 20 $\mu$M) (Fig. 5), suggesting that NtACP binding to $\alpha_1\beta_1$-integrin requires the intact KTD motif. RR', D2-R and BSA showed no significant difference in inhibition ($P>0.1$) from that observed with NtACP D146A. The monoclonal antibody MAB1973 recognizes an epitope located in the $\alpha_1$-integrin I domain (Fabbri *et al.*, 1996). It was included in the assay since it is known to inhibit binding of $\alpha_1$-integrin to laminin and collagen. MAB1973 did not affect ACP binding to $\alpha_1\beta_1$-integrin. Similar assays were performed using polyclonal antibodies to the $\beta_1$ subunits, but no inhibition was observed (data not shown). NtACP may bind to an epitope(s) on $\beta_1$ that is not detectable with the polyclonal antibodies used, or it may bind only to the $\alpha_1$ subunit.
NtACP mediates internalization of GBS via the D1 region

We previously demonstrated that soluble recombinant ACP and NtACP, when added to ME180 cells, bind to the cells and reduce the level of internalized A909 to that achieved with the ACP-deficient isogenic GBS mutant JL2053. RR’ does not affect internalization of either A909 or JL2053 (Bolduc et al., 2002). In addition, structural data suggest that NtACP may bind integrin via a KTD motif in the D1 domain (Auperin et al., 2005). Based on these results and the data presented above, we hypothesize that soluble NtACP may compete for binding to host cell $\alpha_1 \beta_1$-integrin, thus preventing GBS-associated ACP from binding and inhibiting its contribution to the internalization of GBS.

Therefore, in the current study we re-examined the internalization of A909 and JL2053 within ME180 cells in the presence of soluble NtACP D146A, D2 and D2-R to isolate the region within NtACP needed for maximal internalization.

Confluent ME180 cells were incubated with the soluble proteins for 2 h before GBS was added and the cells were allowed to internalize the bacteria. Both NtACP and NtACP D146A reduced A909 internalization, but did not affect the internalization of JL2053 (Fig. 6a). Pretreating cells with 20 $\mu$M NtACP reduced the internalization of A909 by 82% (5.55 times less) compared to that observed with non-treated cells. The single mutation within the KTD motif significantly attenuated NtACP’s ability to inhibit

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Fig. 4. Colocalization of ACP and Inv497 with $\beta_1$-integrin subunit. (a) ACP, (b) Inv497 and (c) BSA were labelled with AlexaFluor 568 (red) and allowed to interact with ME180 cells grown to confluence on transwells. $\beta_1$- and $\beta_2$-integrin subunits were detected with an anti-mouse antibody conjugated to AlexaFluor 488 (green). Colocalization of ACP and Inv497 with $\beta_1$-integrin on the surface of ME180 cells results in yellow fluorescence. (d) ACP labelled with AlexaFluor568 (red) was allowed to interact with ME180 cells grown to confluence on transwells. $\alpha_1$-Integrin was probed with anti-mouse IgG antibodies conjugated to AlexaFluor 488 stained green. Colocalization of ACP with $\alpha_1$-integrin subunit was detected in yellow. (e) ACP labelled with AlexaFluor 568 (red) was allowed to interact with ME180 cells grown to confluence on transwells. $\alpha_1$-Integrin was probed with anti-rabbit IgG antibodies conjugated to AlexaFluor 660 (blue). $\beta_1$-Integrin was probed with anti-mouse IgG antibodies conjugated to AlexaFluor 488 (green). Colocalization of $\alpha_1 \beta_1$-integrin with ACP was detected in white.
A909 internalization in a dose-dependent manner. Cells treated with 20 μM NtACP D146A internalized 61% less GBS than the non-treated cells.

A close structural homology exists between D1 and FnIII10 (Auperin et al., 2005). In addition to FnIII10's RGD integrin-binding motif, FnIII requires two charged residues (Arg1379 and Asp1373) located on module 9 (FnIII9) for maximal integrin binding (Hamburger et al., 1999). D1 residues (Arg110 and Asp118) occupy analogous positions in relation to the KTD motif, suggesting that these residues may also play a direct role in binding ACP to α1β1-integrin. Therefore, mutations within all three residues KTD146, Arg110 and Asp118 may be required to completely prevent the competitive binding of exogenous NtACP and the subsequent entry of wild-type GBS into epithelial cells. The data support the hypothesis that the D1 domain of GBS-associated ACP is required for full GBS internalization into ME180 cells.

The addition of D2 (Fig. 6b) and D2-R (Fig. 6c) resulted in an increase in the percentage of internalized A909 (147.2 ± 7.8 and 158.3 ± 27.3%, respectively). A 30.3% increase in internalized ACP-deficient mutant JL2053 was also observed following the addition of D2-R to the cells. The causes for these observed increases are not understood. We previously demonstrated that GAGs bind D2-R (Auperin et al., 2005). It is possible that D2-R binding to GAGs expressed on the surface of ME180 cells acts as a bridge to stabilize GBS binding and its subsequent internalization within ME180 cells. Alternatively, it is possible that the D2 domain stimulates the cells via an unknown mechanism resulting in the observed increase of GBS internalization.

DISCUSSION

GBS colonizes the large intestine of adults, leading to a possible secondary colonization of the vagina and urethra,
with an estimated average duration of colonization between 8 and 14 weeks (Picard & Bergeron, 2004). Higher frequency is seen among sexually active individuals (Manning et al., 2004; Meyn et al., 2002). GBS colonizing these mucosal sites can traverse the mucosal barriers, resulting in invasive disease. GBS adhesion is required for colonization and subsequent invasion. Several GBS surface proteins bind host extracellular matrix components and serum proteins (Beckmann et al., 2002; Gutekunst et al., 2004; Schubert et al., 2002; Spellerberg et al., 1999). ACP, a virulence factor and prototype for the Alp family of proteins, is the first surface protein identified to bind integral host cell-surface receptors, GAGs and $\alpha_1\beta_1$-integrin. Structural analysis of the GBS C5a peptidase (SCPB) suggests that it too may bind integrins via the RGD motif (Brown et al., 2005), but this has not been demonstrated experimentally.

GBS invasion of epithelial membranes involves internalization within membrane-bound vacuoles and paracellular translocation (Soriani et al., 2006; Valentin-Weigand et al., 1997). GBS internalization, but not translocation, requires actin polymerization and Rho GTPase activity (Baron et al., 2004; Soriani et al., 2006). Capsular expression also attenuates GBS internalization but not translocation (Gibson et al., 1993; Soriani et al., 2006). The different requirements for the two biological events suggest that they may be independent of each other. In contrast, expression of ACP appears to play a role during both events.

We initially reported that ACP binds heparin and epithelial cell-associated GAGs (Baron et al., 2004). NtACP and the repeat region separately do not bind heparin. However, the D2-R peptide consisting of NtACP’s D2 domain covalently associated with a single repeat binds heparin, suggesting that the heparin- and host GAG-binding domain is located within the junction between the D2 domain and the adjacent repeat. The NtACP tertiary structure maps a portion of the putative heparin-binding domain to BR2, a positively charged cluster within the D2 domain, adjacent to the first repeat region (Auperin et al., 2005). We now report that ACP binds to a second cell-surface receptor, $\alpha_1\beta_1$-integrin, via the D1 domain located on the opposite side of the molecule from the putative GAG-binding domain. The $\alpha_1\beta_1$-integrin is one of four collagen-binding I-domain-containing integrins (Gullberg & Lundgren-Akerlund, 2002). It preferentially binds to collagen IV but also binds collagen I and laminin via the I domain (Calderwood et al., 1997; Riikonen et al., 1995). The monoclonal antibody MAB1973 recognizes an epitope specific to the human $\alpha_1$-integrin I domain and inhibits binding of activated human lymphocytes to these extracellular matrix components (Fabbri et al., 1996). MAB1973 does not affect ACP binding to $\alpha_1\beta_1$-integrin. However, the presence of a polyclonal antibody to $\alpha_1$-integrin does reduce ACP binding to $\alpha_1\beta_1$-integrin by 42%, suggesting that ACP binding to $\alpha_1$-integrin differs from that of collagen and laminin. One possible explanation is that ACP binds to $\alpha_1$-integrin via a unique non-I-domain interaction.

Proteins of other micro-organisms have similarly been reported to bind GAGs and integrin as co-receptors. For example, fibronectin acts as a bridge binding OpA, a Neisseria gonorrhoeae outer-membrane protein, to $\alpha_5\beta_1$-integrin and is essential for N. gonorrhoeae internalization (van Putten et al., 1998). GAG forms a complex with fibronectin and integrin for maximal internalization. The Borrelia burgdorferi surface lipoprotein BBK32 also binds fibronectin and GAG (Fischer et al., 2006). ACP differs such that it binds directly to the integrin molecule without the use of a bridge. Pretreating ME180 cells with sodium chloride, an inhibitor of sulfate incorporation, or with heparinase inhibits binding of ACP to the cells by 84–98% (Baron et al., 2004). We currently report that a single mutation within the KTD motif (D146A), present in the D1 domain, reduces NtACP binding to $\alpha_1\beta_1$ and ME180 by 20–25%. Therefore, we sought to determine the contribution of integrin binding to GBS internalization within host cells.

Mutagenesis of NtACP, generating a KTD146A single mutation within the putative KTD integrin-binding motif, reduced its ability to inhibit GBS internalization. In similar assays where ME180 cells were preincubated with D2-R, containing the putative GAG-binding domain, internalization was not diminished, but instead increased. This effect does not require the expression of ACP on GBS. The RR’ peptide had no measurable effect on GBS internalization. The data suggest that binding to both GAG and integrin may be needed for maximal binding of ACP to ME180 cells. However, the direct interaction between D1 of ACP and $\alpha_1\beta_1$-integrin plays a key role in GBS internalization.

Structural analysis of the D1 domain, in particular the region containing the putative integrin-binding loop and KTD motif, shares a strong structural homology with the FnIII10’s integrin-binding region. Amino acid sequence alignment of Alps indicates that KTD is highly conserved (Auperin et al., 2005; Creti et al., 2004), suggesting biological importance. The RGD motif present on FnIII10 interacts directly with integrin and is the canonical integrin-binding motif. Sixteen other motifs, not including KTD, were identified on short disintegrins (Sanz et al., 2006). Each motif binds to a specific integrin heterodimer. The first two residues of the triads dictate the specificity of the $\alpha$ subunit (Sanz et al., 2006). The motif KTS is expressed by obtustatin and viperistatin and binds $\alpha_2\beta_1$, in a $\alpha$-domain-independent manner, similar to ACP (Marciniec-wicz et al., 2003). We show that ACP interacts with the $\alpha_1$ subunit of $\alpha_1\beta_1$-integrins. Also, the KT(D146A) substitution reduces the binding of NtACP to $\alpha_1\beta_1$-integrins. ACP did not alter binding of a polyclonal antibody specific to the $\beta_1$ subunit in similar experiments (data not shown). ACP may alter binding of a different source of poly- or monoclonal antibodies to $\beta_1$-integrin. Therefore, we cannot rule out the possibility that ACP also binds to the $\beta_1$ subunit.

The $\alpha_1\beta_1$-integrin is mainly expressed by mesenchymal cells and activated T lymphocytes and monocytes (Ben-Horin & Bank, 2004; Duband et al., 1992; Hemler, 1990; Rubio et al.,...
inducible expression of the adhesion molecules VCAM-1 and ICAM-1. Am J Respir Cell Mol Biol 17, 571–582.


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